Identification of the Regulatory Subunit of *Arabidopsis thaliana* Acetohydroxyacid Synthase and Reconstitution with Its Catalytic Subunit†

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ABSTRACT: Acetohydroxyacid synthase (EC 4.1.3.18; AHAS) catalyzes the initial step in the formation of the branched-chain amino acids. The enzyme from most bacteria is composed of a catalytic subunit, and a smaller regulatory subunit that is required for full activity and for sensitivity to feedback regulation by valine. A similar arrangement was demonstrated recently for yeast AHAS, and a putative regulatory subunit of tobacco AHAS has also been reported. In this latter case, the enzyme reconstituted from its purified subunits remained insensitive to feedback inhibition, unlike the enzyme extracted from native plant sources. Here we have cloned, expressed in *Escherichia coli*, and purified the AHAS regulatory subunit of *Arabidopsis thaliana*. Combining the protein with the purified *A. thaliana* catalytic subunit results in an activity stimulation that is sensitive to inhibition by valine, leucine, and isoleucine. Moreover, there is a strong synergy between the effects of leucine and valine, which closely mimics the properties of the native enzyme. The regulatory subunit contains a sequence repeat of approximately 180 residues, and we suggest that one repeat binds leucine while the second binds valine or isoleucine. This proposal is supported by reconstitution studies of the individual repeats, which were also cloned, expressed, and purified. The structure and properties of the regulatory subunit are reminiscent of the regulatory domain of threonine deaminase (EC 4.2.1.16), and it is suggested that the two proteins are evolutionarily related.

The biosynthesis of valine and leucine involves a series of reactions in which two molecules of pyruvate are converted to one of 2-ketoisovalerate. This may then be converted to valine by transamination, or react with acetyl CoA and undergo a further series of conversions to 2-ketoisocaproate that is then transaminated to leucine. The biosynthesis of the third branched-chain amino acid, isoleucine, parallels that of valine except that the pathway starts with one molecule each of pyruvate and 2-ketobutyrate rather than two molecules of pyruvate. The first step in all of these processes is the reaction catalyzed by acetohydroxyacid synthase [AHAS[1]; EC 4.1.3.18; see Chipman et al. (*1*) and Duggleby and Pang (*2*) for reviews]. This thiamin diphosphate-dependent enzyme catalyzes the decarboxylation of pyruvate to an enzyme-bound hydroxyethyl group that then condenses with either pyruvate or 2-ketobutyrate to form 2-acetolactate or 2-aceto-2-hydroxybutyrate, respectively.

AHAS from most, and perhaps all, bacteria is composed of a large subunit that contains the main catalytic machinery, and a smaller regulatory subunit. The best studied examples of bacterial AHASs are those from the enterobacteria, where there are three isoforms. In these enzymes, the catalytic subunit alone possesses low (*3*, *4*) or no (*5*) activity, but reconstitution in vitro with the regulatory subunit restores full enzymatic activity. In addition, regulation by the feedback inhibitor valine is not observed for the catalytic subunit alone but is restored in the reconstituted system. The exception is *Escherichia coli* isoenzyme II, which is intrinsically insensitive to valine (*5*, *6*).

The subunit composition of eucaryotic AHASs is less clear. A gene corresponding to the catalytic subunit has been found and sequenced in a variety of fungi and plants. Some of these have been expressed in *E. coli* and shown to be active, but in all cases the activity is insensitive to inhibition by branched-chain amino acids (*7*−*12*). In contrast, the eucaryotic enzyme extracted from its native source is inhibited by one or more of the branched-chain amino acids (*7*, *13*−*21*). This insensitivity to inhibition of the expressed fungal and plant enzymes is one of the lines of evidence that favors the existence of a eucaryotic AHAS regulatory subunit (*22*).

An open reading frame discovered in yeast was proposed to be an AHAS regulatory subunit (*22*, *23*), and this hypothesis was later confirmed by in vitro reconstitution with the yeast catalytic subunit (*12*). Combining the two purified subunits results in a 7−10-fold stimulation of the activity of the catalytic subunit and confers upon it sensitivity to valine inhibition. These experiments provided the first unequivocal proof for a eucaryotic AHAS regulatory subunit. For the plant enzyme, efforts to identify a regulatory subunit have been less successful. Hershey et al. (*24*) reported a possible candidate in curled-leaved tobacco (*Nicotiana plumbaginifolia*); while stimulation of the activity of the catalytic subunit was demonstrated upon combining it with the putative regulatory subunit, no effect of branched-chain amino acids was observed.

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‡ Yu-Ting Lee disagrees with any statement or implication that the relationships between proteins mentioned in this paper are a result of evolution or support the theory of evolution.

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[1] Abbreviations: AHAS, acetohydroxyacid synthase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; bp, base pairs.

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Table 1: Oligonucleotides Used for Sequencing and Cloning

name[a]	sequence[b]
T7 term	5′-GCTAGTTATTGCTCAGCGG-3′
ARSU-51	5′-TG*cc*ATGGGTAAGAGAATGGAAGGATTCG-3′
ARSU-70	5′-TC*gaattcat*GTCTTTCTCCGAAGCTTCATCTGC-3′
ARSU-85	5′-CG*gAatTc*AtGAAGCACACAATTTCAGTATTTGTTGG-3′
T7 prom	5′-TAATACGACTCACTATAGG-3′
ARSU-1220	5′-AGCAGCAGCGTTCACGGC-3′

[a] The T7 promotor and ARSU-1220 oligonucleotides were used as primers for sequencing reactions. Other oligonucleotides were used as PCR primers. [b] Bases shown in lower case are those that are different from the wild-type sequence. Underlined italics indicate the location of *Nco*I (in ARSU-51) or *Eco*RI (in ARSU-70 and ARSU-85) sites.

In this paper, we report the identification of the regulatory subunit of *Arabidopsis thaliana* AHAS. In vitro reconstitution results in enhanced activity of the catalytic subunit that also acquires sensitivity to regulation by the branched-chain amino acids. Moreover, unlike the bacterial enzyme, the reconstituted *A. thaliana* enzyme is inhibited by all three branched-chain amino acids, but especially by the combination of leucine plus valine. These properties match closely those that have been described for the plant enzyme extracted from its native source (*14*, *16*, *19*). The *A. thaliana* AHAS regulatory subunit amino acid sequence shows clear evidence of an internal duplication, and we suggest that the protein contains two domains with one binding valine (or isoleucine) and the other leucine. Further, it is suggested that there is an evolutionary relationship to the regulatory domain of threonine deaminase (EC 4.2.1.16), which also contains an internal duplication that creates separate binding sites for its regulators valine and isoleucine (*25*).

EXPERIMENTAL PROCEDURES

Materials and experimental procedures were as described previously (*26*), except as noted below. The *A. thaliana* EST clone G7C11T7 was obtained from the Arabidopsis Biological Resource Center, DNA Stock Center, The Ohio State University, Columbus, Ohio. Methods for bacterial culture and DNA manipulation generally followed the procedures of Sambrook et al. (*27*).

Cloning. The EST clone G7C11T7 was digested with *Eco*RI and ligated to pET30b(+) that had also been digested with *Eco*RI. This construct (pET30b(+)·G7C11T7) expresses a protein consisting of the first 45 amino acids common to all the pET30 vectors (including a hexahistidine tag), a connector with the sequence AISDPNSRPRFVSS, followed by the full length (491 amino acids) of the *A. thaliana* AHAS regulatory subunit.

Three plasmids with various truncations of the chloroplast transit peptide coding sequence were constructed by PCR. In each case, the template was pET30b(+)·G7C11T7, the reverse primer was the T7 terminator oligonucleotide (Table 1), and PCR conditions were 95 °C for 30 s, 30 cycles of (95 °C for 30 s, 60 °C for 1 min, and 72 °C for 2 min), followed by 72 °C for 7 min. For the first plasmid (pET30a(+)·ARSU-51), the forward primer used was ARSU-51 (Table 1), and the resulting product was cloned into pET30a(+) as an *Nco*I/*Not*I fragment. The expressed protein consists of the first 44 amino acids of the pET30 vector followed directly by the complete sequence of the *A. thaliana* AHAS regulatory subunit from M52 to G491. For the second plasmid (pET30a(+)·ARSU-70), the forward primer used was ARSU-70 (Table 1), and the resulting product was cloned into pET30a(+) as an *Eco*RI/*Not*I fragment. The expressed protein consists of the first 45 amino acids of the pET30 vector, a connector with the sequence ADIGSEFM, followed directly by the complete sequence of the *A. thaliana* AHAS regulatory subunit from S71 to G491. For the third plasmid (pET30a(+)·ARSU-85) the forward primer used was ARSU-85 (Table 1), and the resulting product was cloned into pET30a(+) as an *Eco*RI/*Eco*RI fragment. The expressed protein has the same amino acid sequence as that expressed by pET30a(+)·ARSU-70, except that the part derived from the *A. thaliana* AHAS regulatory subunit starts at K86 instead of S71.

Plasmids expressing the two separate repeats of the regulatory subunit were constructed by taking advantage of a *Bgl*II site at the 3′-end of the first repeat. For the first (pET30a(+)·ARSU·R1), pET30a(+)·ARSU-70 was digested with *Bgl*II and the 615 bp fragment so obtained was cloned into the *Bgl*II site of pET30a(+). The expressed protein has the same amino acid sequence as that expressed by pET30a(+)·ARSU-70 except that the *A. thaliana* AHAS regulatory subunit terminates at L258 and is followed by the tail sequence GTDDDDKAMADIGSEFELRRQACGRTRAPPPPPLRSGC. For the second (pET30a(+)·ARSU·R2), pET30a(+)·ARSU-70 was digested with *Bgl*II and *Not*I, and the 859-bp fragment so obtained was cloned into pET30a(+) digested with *Bgl*II and *Not*I. The expressed protein consists of the first 33 amino acids of the pET30 vector followed directly by the complete sequence of the *A. thaliana* AHAS regulatory subunit from P256 to G491.

All constructs were tested by diagnostic restriction enzyme digestion. The complete DNA sequence of the insert in pET30b(+)·G7C11T7 was determined using the Prism Ready Dye Terminator Cycle Sequencing kit and DNA Sequencer 373A (Perkin-Elmer Applied Biosystems, Norwalk, CT).

Expression and Purification of AHAS Subunits. Expression and purification of the *A. thaliana* AHAS catalytic subunit was as described previously (*26*). Expression and purification of various forms and fragments of the regulatory subunit were by an adaptation of the method of Lee and Duggleby (*28*) for *E. coli* AHAS isoenzyme II. The main difference is that buffers did not include FAD, and purification by immobilized-metal affinity chromatography involved a wash with 60 mM imidazole, followed by elution using an imidazole gradient from 60 to 400 mM. Fractions containing the desired protein were pooled and concentrated by ultrafiltration.

Enzyme and Protein Assays. The standard assay for AHAS enzymatic activity was performed in 200 mM potassium phosphate buffer (pH 7.0) containing 200 mM pyruvate, cofactors (1 mM thiamin diphosphate, 10 mM $MgCl_2$, and 10 μM FAD), inhibitors (where added), and catalytic and regulatory subunits as required for the particular experiment. The subunits in buffer were incubated at 30 °C for 10 min to allow reconstitution to occur, and then cofactors were added and incubation at 30 °C was continued for a further 10 min. The reaction was initiated by the simultaneous addition of pyruvate and inhibitors and allowed to continue for 30 min at 30 °C. Acetolactate so formed was converted to acetoin and estimated colorimetrically using the method of Westerfeld (*29*) as described previously (*26*). One unit is

defined as the amount of enzyme that produces 1 μmol of acetolactate/min. For some experiments, the preincubation with cofactors was omitted, and these were added simultaneously with the substrate.

Protein was estimated using bicinchoninic acid (*30*), and SDS−PAGE was performed as described by Laemmli (*31*).

Gel-Filtration Chromatography. Gel-filtration chromatography experiments were carried out at room temperature on a Superdex HR 10/30 column (Pharmacia Biotech) connected to an FPLC System (Pharmacia Biotech). The column was equilibrated with a buffer consisting of 50 mM potassium phosphate (pH 7.0), 0.15 M NaCl, 15% glycerol, 10 μM FAD, and 1 mM dithiothreitol. Protein samples were loaded onto the column via a 50 μL sample loop and eluted at a flow rate of 0.5 mL/min. The elution of the protein was monitored by following the absorbance at 280 nm. The column was calibrated with six protein standards (Sigma): bovine thyroglobulin (669 kDa), horse apoferritin (443 kDa), sweet potato β-amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and bovine carbonic anhydrase (29 kDa). A molecular mass calibration curve was constructed by plotting the logarithm of the protein molecular mass against the ratio of the elution volume to void volume. The AHAS subunit composition of eluted peaks was assessed by SDS−PAGE, and quantified by densitometry of these gels using standards containing known amounts of the catalytic and regulatory subunits.

Data Analysis. Where appropriate, data were analyzed by nonlinear regression, fitting the chosen equation to the data using the program GraFit (Erithacus Software Ltd., Staines, UK).

RESULTS

Sequencing of the *A. thaliana* genome has provided the opportunity to identify genes that have not been discovered by conventional biochemical or genetic studies. Database searching of GenBank revealed a candidate gene for the *A. thaliana* AHAS regulatory subunit on chromosome 2 (accession number AC006533). In silico transcription, splicing, and translation (*32*) yielded a potential protein of 484 amino acids that shows high homology to the putative *N. plumbaginifolia* AHAS regulatory subunit (*24*). Further database searching identified two *A. thaliana* EST clones (G7C11T7 and 157J23T7) containing at least part of the DNA that would encode this subunit. One of these (G7C11T7) was subcloned into pET30b(+) and completely sequenced (Figure 1A). This sequence is identical to that predicted from the in silico analysis of genomic DNA except at two places. The first is at the junction of the fifth and sixth exon where the last 9 bp of exon 5 are absent so that the actual amino acid sequence is TGKIAL rather than TGKVVYIAL. The second is at the junction of the eighth and ninth exon where exon 8 extends for a further 30 bp, thereby adding the amino acid sequence VFARRGYNIQ. As a result of these two differences, the encoded protein has a length of 491 amino acids. In all cases, the introns would have the expected 5′-GU⋯⋯AG-3′ sequence that is found at most eucaryotic splice sites.

The amino acid sequence of the *A. thaliana* AHAS regulatory subunit consists of four regions (Figure 1B). The first 80 residues have a composition and sequence that is typical of chloroplast targeting peptides (*33*); this is expected because it is known that AHAS activity is found in chloroplasts (*34*). Moreover, this sequence has a strong similarity to the corresponding region of the *A. thaliana* AHAS catalytic subunit (Figure 1C). The transit peptide is followed by two duplicate regions of ~180 residues separated by a ~50 residue linker (Figure 1B). The similarity between the duplicate regions is illustrated in Figure 1D. The overall structure is similar to that described for the putative *N. plumbaginifolia* AHAS regulatory subunit (*24*).

Expression and Purification. The initial construct tested for expression contains the full-length regulatory subunit plus a 59-residue N-terminal fusion peptide incorporating a hexahistidine sequence (6H/ARSU, Figure 2). The level of expression found using this construct was very low, so other constructs were tested in which the chloroplast transit peptide region was truncated (Figure 2). Removal of either 51 or 70 residues results in greater expression and a soluble protein, while removal of 85 residues gives a low level of expression. The protein with 70 residues removed (6H/ARSU-70) was used for most of the experiments reported below. Even though the protein is soluble, its solubility is low, and this limits the experiments that can be performed with the protein.

The protein was purified by immobilized-metal affinity chromatography and appeared to be close to purity as judged by SDS−PAGE (Figure 3). The estimated molecular mass is 52.9 kDa, in good agreement with the calculated value of 52.4 kDa.

Activation of the Catalytic Subunit. Mixing of the catalytic and regulatory subunits results in a time-dependent increase in activity (data not shown). After approximately 5 min, activity reaches a plateau, and, for all subsequent experiments with the reconstituted enzyme, the subunits were preincubated together for a minimum of 8 min. The activity shows a hyperbolic dependence on the concentration of regulatory subunit (Figure 4) with a maximum observed activation of 5.4-fold in this experiment. Extrapolation of the curve indicated an asymptote at 7.5-fold activation, but this limit could not be reached in practice due to the limited solubility of the regulatory subunit. The half-saturating concentration of the regulatory subunit calculated from these data is 167 ± 24 nM, which is interpreted as the dissociation constant for the complex formed between the two types of subunit. Unlike yeast AHAS, which requires a high (~1 M) phosphate concentration (*12*), reconstitution of the *A. thaliana* enzyme from its subunits occurs readily at 0.2 M phosphate and is largely unaffected by phosphate concentrations in the range of 0.05 to 0.4 M (data not shown).

The subunit composition of reconstituted AHAS was assessed by gel-filtration chromatography followed by SDS−PAGE. The major eluted peak (Figure 5A) has an apparent molecular mass of approximately 548 kDa and contains both subunits. Quantitative densitometry of the reconstituted enzyme (Figure 5B) gave a catalytic/regulatory subunit molar ratio of 1.17 and of 1.11 in two experiments. We interpret these data to indicate that the reconstituted enzyme contains equal numbers of catalytic and regulatory subunits. The molecular masses of the catalytic and regulatory subunit are 65.1 and 52.4 kDa, respectively, so the composition of the major peak observed in gel-filtration (548 kDa) is consistent with four or five copies of each subunit in the complex.

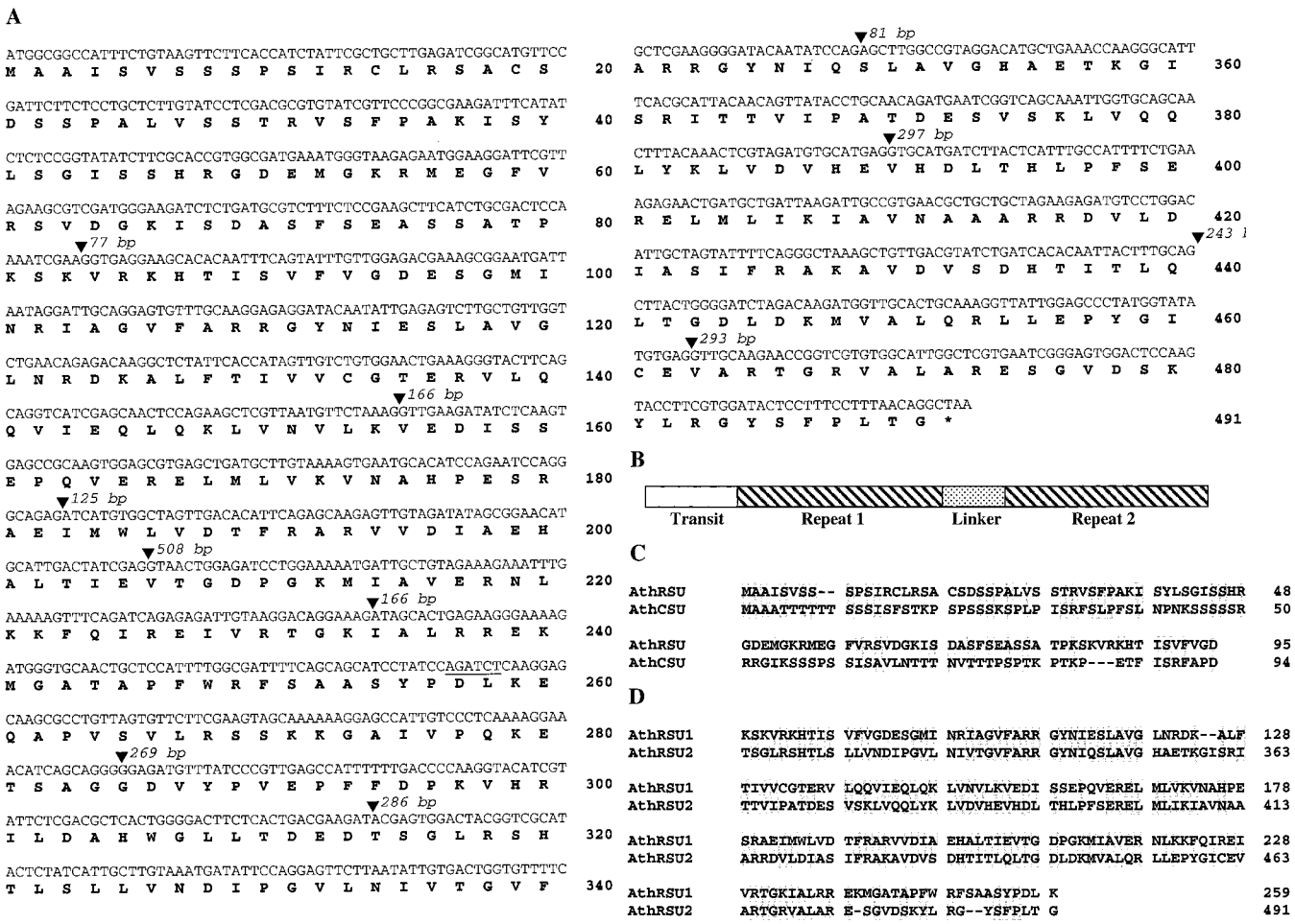

FIGURE 1: Sequence analysis of the *A. thaliana* AHAS regulatory subunit. Panel A shows the cDNA sequence and the deduced amino acid sequence. Introns within the open reading frame are shown as ▼ together with their size in base pairs. The *Bgl*II site that was used to separately subclone the first and second repeat is underlined. Panel B shows a schematic representation of the protein. Panel C shows an alignment between the N-terminal regions of the regulatory (AthRSU) and catalytic (AthCSU) subunits of *A. thaliana* AHAS. Panel D shows an alignment between the first (AthRSU1) and second (AthRSU2) repeats of the *A. thaliana* AHAS regulatory subunit. In panels C and D, gray shading indicates residues conserved between pairs of compared sequences. Conserved residues are defined as those that are identical between the two sequences, or are members of one of the strong conservation groups (STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, and FYW).

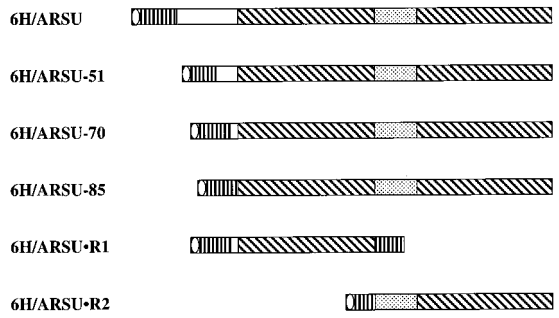

FIGURE 2: Schematic representation of the structures of the proteins expressed. Sequences derived from the *A. thaliana* regulatory subunit are shaded as in Figure 1, while those derived from the pET30 expression vector are shaded by vertical bars. The white oval at the extreme N-terminus represents the hexahistidine tag.

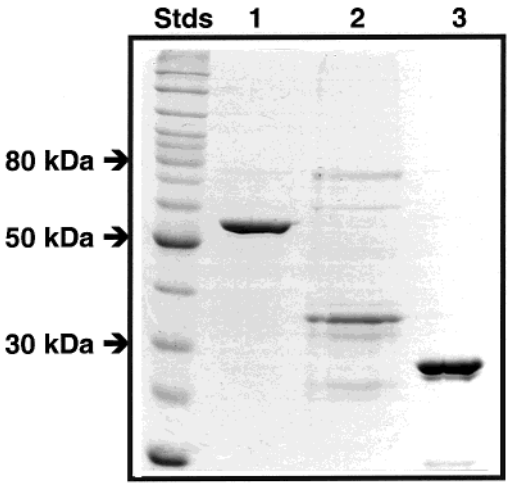

FIGURE 3: SDS−PAGE analysis of the purified regulatory subunit of *A. thaliana* AHAS. The leftmost lane contains a ladder of protein standards, while the remaining lanes contain the proteins designated in Figure 2 as 6H/ARSU-70, lane 1; 6H/ARSU•R1, lane 2; and 6H/ARSU•R2, lane 3.

The activated enzyme shows an hyperbolic substrate saturation curve (Figure 6) with a Michaelis constant of 11.7 ± 0.6 mM. This is somewhat different to the catalytic subunit alone (*9*), which shows negative cooperativity toward the substrate (Hill coefficient of 0.60 ± 0.04) and a half-saturating substrate concentration of 17.2 ± 3.0 mM. Thus, reconstitution with the regulatory subunit abolishes the negative cooperativity but otherwise has a small effect only on the substrate saturation curve.

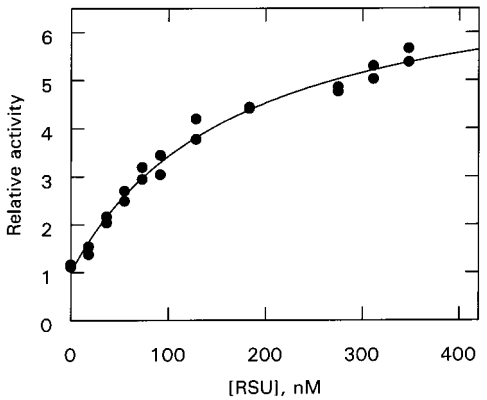

FIGURE 4: Activation of the *A. thaliana* AHAS catalytic subunit by its regulatory subunit. Mixtures containing 13 nM catalytic subunit and various concentrations of regulatory subunit in 200 mM potassium phosphate buffer (pH 7.0) were preincubated at 30 °C for 8 min. The reaction was started by the simultaneous addition of 50 mM pyruvate and cofactors. Rates are expressed relative to the specific activity with no added regulatory subunit. The line represents a curve fitted to the data using the equation: activity = $1 + (V_\infty - 1)[\mathrm{RSU}]/(K_d + [\mathrm{RSU}])$. The best-fit parameters obtained were $V_\infty = 7.48 \pm 0.32$ and $K_d = 167 \pm 24$ nM.

Branched-Chain Amino Acid Inhibition. We (*9*, *12*) and others (*7*, *8*, *10*, *11*) have noted previously that the catalytic subunit of eucaryotic AHASs, expressed in *E. coli*, is insensitive to inhibition by branched-chain amino acids. We tested the reconstituted *A. thaliana* system for sensitivity to valine, leucine, isoleucine, and to various combinations (Table 2). Each amino acid was found to be inhibitory, and there is some degree of synergy when leucine is combined with valine or isoleucine. For example, the combination of leucine and valine (each at 0.5 mM) gives greater inhibition (65%) than either leucine (49%) or valine (36%) each at 1 mM. Apart from being slightly less sensitive to valine either alone or in combination with isoleucine, the extent of inhibition is similar to the values observed for *A. thaliana* AHAS extracted from its native source (Table 2).

Saturation curves for each of the individual branched-chain amino acids are shown in Figure 7A, and the combination of leucine plus valine in Figure 7B. In all cases, inhibition is partial so that a saturating concentration of inhibitor results in 35−55% residual activity. Apparent inhibition constants for each of the individual amino acids are in the millimolar range (valine, 0.231 ± 0.074 mM; leucine, 0.336 ± 0.030 mM; isoleucine, 1.38 ± 0.16 mM). Although comparison of the apparent inhibition constants would suggest that valine is a slightly more potent inhibitor than leucine, this does not allow for the fact that leucine gives 63% inhibition at saturation, as compared to 45% for valine. The inhibition curves cross at 21 μM (where there is a mere 4% inhibition), and the amount of inhibition by leucine is greater than that caused by valine at all concentrations above 21 μM (e.g., Table 2).

The combination of leucine plus valine exhibits a substantial synergy, resulting in an apparent inhibition constant of 12.3 ± 2.5 μM (Figure 7B). There is some synergy with the combination of isoleucine plus leucine (apparent K_i of 0.194 ± 0.035 mM) but not for valine plus isoleucine (data not shown). For this latter combination, the apparent K_i of 0.482 ± 0.053 mM is indistinguishable statistically from the harmonic mean of the values for the individual inhibitors

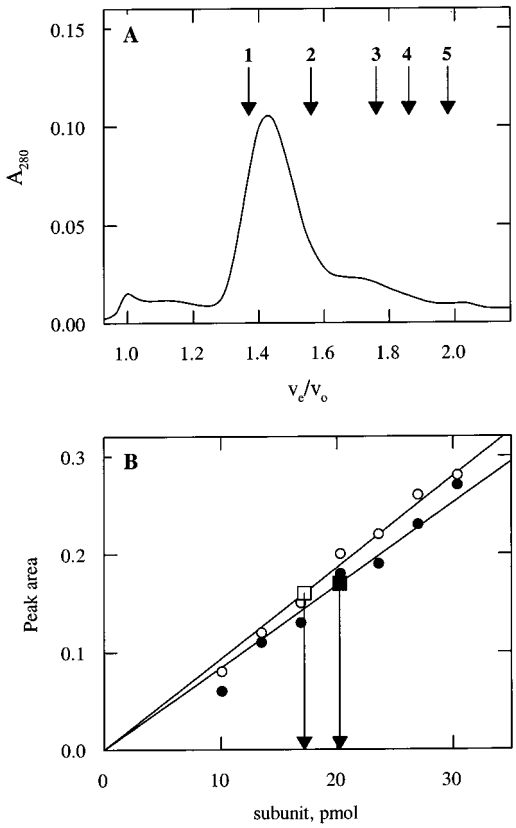

FIGURE 5: Gel-filtration chromatography and quantitative densitometry of reconstituted *A. thaliana* AHAS. The reconstituted enzyme was subjected to gel-filtration chromatography (panel A), and the apparent molecular mass of 548 kDa was estimated by comparison to the elution volume of bovine thyroglobulin (arrow 1; 669 kDa), horse apoferritin (2; 443 kDa), sweet potato α-amylase (3; 200 kDa), yeast alcohol dehydrogenase (4; 150 kDa), bovine serum albumin (5; 66 kDa), and bovine carbonic anhydrase (not shown; 29 kDa). The elution volume (v_e) is plotted relative to the void volume (v_o). The peak fractions were collected and the AHAS subunits were analyzed by SDS−PAGE. These were then quantified using densitometry by comparison with known amounts of regulatory (○) and catalytic (●) subunit. From the measured peak area of the regulatory (□) and catalytic (■) subunit, the reconstituted enzyme was estimated to contain 17.2 and 20.2 pmol, respectively, of the two subunits.

(0.396 ± 0.095 mM), as expected if valine and isoleucine are competing for the same binding site.

The synergy between valine and leucine was investigated further by varying the concentration of leucine in the presence of a constant concentration of valine (Figure 7C). The apparent K_i for leucine falls from 313 ± 78 μM (in the absence of valine) to 44.9 ± 7.8, 11.0 ± 1.7, and 2.6 ± 0.6 μM, on addition of 5, 10, and 20 μM valine, respectively. Very strong synergy is observed; for example, addition 20 μM valine, which alone is barely inhibitory, causes a 120-fold decrease in the apparent K_i for leucine. Complementary experiments varying the valine concentration in the presence of leucine gave a similar picture (data not shown). For example, addition of 10 and 20 μM leucine resulted in apparent K_i values for valine of 6.8 ± 1.6 μM and 5.3 ± 0.6 μM, as compared to the value of 231 ± 74 μM observed in the absence of leucine.

The activation by the regulatory subunit, and the sensitivity of the reconstituted enzyme to leucine inhibition, is pH-dependent (Table 3). There are no major effects in the pH

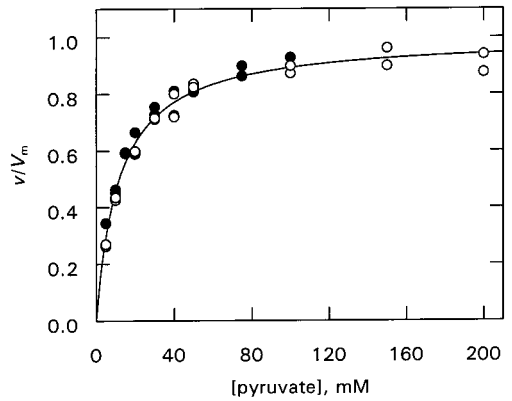

FIGURE 6: Substrate saturation curve of reconstituted *A. thaliana* AHAS. Mixtures containing 6.6 nM catalytic subunit and 513 (●) or 912 (○) nM regulatory subunit in 200 mM potassium phosphate buffer (pH 7.0) were preincubated at 30 °C for 15 min. The reaction was started by the addition of cofactors, and various concentrations of pyruvate as shown. The two experiments gave slightly different maximum velocities (V_m), so rates have been normalized to a common ordinate scale. The line represents a curve fitted to the data using the Michaelis−Menten equation, and corresponds to a Michaelis constant of 11.7 ± 0.6 mM.

Table 2: Inhibition of *A. thaliana* AHAS by Branched-Chain Amino Acids

addition[a]	percent inhibition	
	reconstituted[b]	native[c]
valine	36	47
leucine	49	52
isoleucine	27	33
valine + leucine	65	67
valine + isoleucine	35	50
leucine + isoleucine	61	64

[a] At a concentration of 1 mM for each individual amino acid, or 0.5 mM each for the combinations. [b] Using 24 nM catalytic subunit and 435 nM regulatory subunit. [c] Taken from Stidham and Singh (*35*).

range from 6.0 to 7.0, but at higher pH values there is less activation by the regulatory subunit, and the sensitivity to leucine decreases so that at a pH value of 7.8, there is slight inhibition only.

Reconstitution with the First and Second Regulatory Subunit Repeats. To investigate further the roles of the first and second repeat regions of the regulatory subunit, expression plasmids were constructed containing the coding sequence for only one of these repeats. The first (6H/ARSU·R1, Figure 2) contains residues 71 to 258, while the second (6H/ARSU·R2) contains residues 256 to 491. Expression of either of these subunit fragments gave proteins that are largely insoluble, but there was sufficient soluble material to be purified by immobilized-metal affinity chromatography. As shown in Figure 3, the resulting purified fragments constituted the major protein band in SDS−PAGE analysis.

Each was tested for its ability to activate the *A. thaliana* AHAS catalytic subunit and the ability to confer upon it sensitivity to branched-chain amino acid inhibition. Each of the repeats is capable of activating the catalytic subunit (Figure 8), although neither is as effective as the entire regulatory subunit. This is due, at least in part, to the difficulty in providing sufficient amounts of each repeat due to their limited solubility. The enzyme reconstituted with the first repeat was inhibited by leucine (48% at 10 mM) but not by valine or isoleucine, and no synergy was observed with combinations of the branched-chain amino acids. The enzyme reconstituted with the second repeat was not inhibited by any of the branched-chain amino acids.

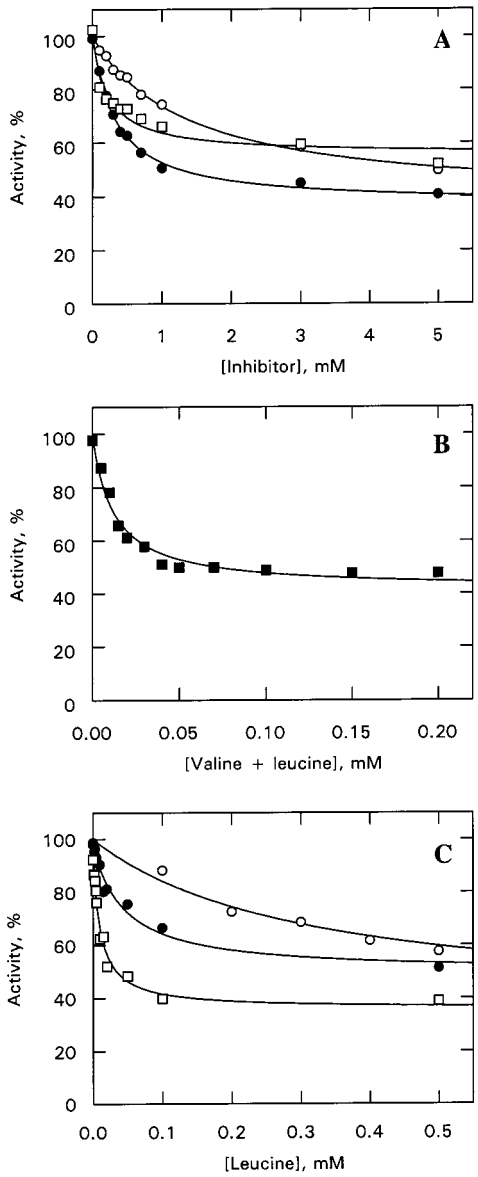

FIGURE 7: Inhibition of reconstituted *A. thaliana* AHAS by branched-chain amino acids. Mixtures containing 24 nM catalytic subunit and 435 nM regulatory subunit in 200 mM potassium phosphate buffer (pH 7.0) were preincubated at 30 °C for 10 min. Cofactors were added and incubation continued for a further 10 min before starting the reaction by the simultaneous addition of 200 mM pyruvate and amino acid inhibitors. Rates are expressed as a percentage of the specific activity with no added amino acids. Panel A shows the effect of individual branched-chain amino acids, panel B shows the effect of added equimolar concentrations of valine + leucine, and panel C shows the effect of added leucine in the presence of 0 (○), 5 (●), and 10 (□) μM valine. The lines represent curves fitted to the data using the equation: activity = $A_\infty + (A_0 - A_\infty)/(1 + [I]/K_i)$ where A_0 is set at 100% for the lines in panel A, panel B, and the control (○) in panel C. The quantity [I] represents the concentration of the varied amino acid, or the sum of [valine] + [leucine] in panel B. The best-fit parameters obtained are panel A, isoleucine (○), A_∞ = 37.0 ± 2.5%, K_i = 1.38 ± 0.16 mM; leucine (●), A_∞ = 36.7 ± 1.3%, K_i = 0.336 ± 0.030 mM; valine (□), A_∞ = 55.3 ± 3.0%, K_i = 0.231 ± 0.074 mM; panel B, valine + leucine (■), A_∞ = 41.3 ± 2.3%, K_i = 12.3 ± 2.3 μM; panel C, no valine (○), A_∞ = 37.0 ± 2.5%, K_i = 1.38 ± 0.16 mM; 5 μM valine (●), A_∞ = 34.3 ± 5.7%, K_i = 313 ± 78 μM; 10 μM valine (□), A_∞ = 35.8 ± 1.7%, K_i = 11.0 ± 1.7 μM.

Table 3: Effect of pH on *A. thaliana* AHAS

pH	change in activity: stimulation (fold)[a]	change in activity: inhibition (%)[b]
6.0	4.9	41
6.5	4.3	42
7.0	4.5	44
7.5	4.0	24
7.8	3.6	8

[a] Using 24 nM catalytic subunit and 392 nM regulatory subunit. [b] Inhibition by 5 mM leucine.

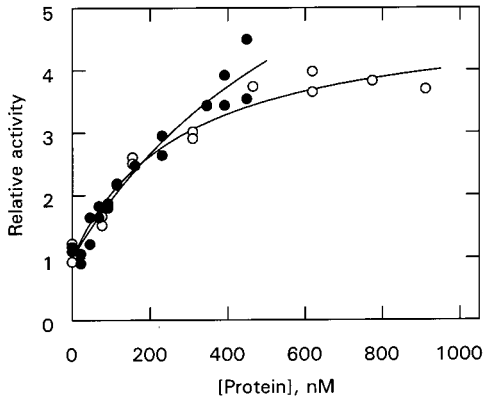

FIGURE 8: Activation of the *A. thaliana* AHAS catalytic subunit by individual regulatory subunit repeats. Mixtures containing 6.6 nM catalytic subunit and various concentrations of regulatory subunit repeats 1 (●) and 2 (○) were treated, and the data analyzed, as described in Figure 4 except that for repeat 1 the preincubation was for 15 min. The best-fit parameters obtained were repeat 1, $V_\infty = 8.81 \pm 1.73$ and $K_d = 740 \pm 234$ nM; repeat 2, $V_\infty = 4.90 \pm 0.35$ and $K_d = 278 \pm 65$ nM.

DISCUSSION

Following leads provided by genetic evidence (*36*) and DNA sequencing (*37*), it was shown by Eoyang and Silverman (*38*) that *E. coli* AHAS isoenzyme I contains two type of subunit with the smaller type playing a regulatory role. Subsequently, regulatory subunits have been identified for most bacterial AHASs (*2*). However, until 1999, the existence of a eucaryotic AHAS regulatory subunit had been conjectured (*22*) but not proven.

In vitro reconstitution studies of yeast AHAS (*12*) provided the first unequivocal evidence for a regulatory subunit in any eucaryotic system. Combining the purified regulatory and catalytic subunits stimulated the activity of the latter by 7−10-fold and conferred upon it sensitivity to feedback inhibition by valine. Shortly afterward, Hershey et al. (*24*) described a putative plant AHAS regulatory subunit in *N. plumbaginifolia*. While in vitro reconstitution stimulated AHAS activity, the enzyme remained unaffected by branched-chain amino acids. Since the insensitivity of the catalytic subunit to branched-chain amino acids is one of the main reasons for suspecting the existence of a regulatory subunit (*22*), the failure to restore this property left some doubt as to whether the *N. plumbaginifolia* protein was really the missing subunit.

In this study, we have reconstituted *A. thaliana* AHAS from its catalytic and regulatory subunits. Combining the subunits stimulated the activity of the catalytic subunit and, more importantly, conferred upon it sensitivity to inhibition by branched-chain amino acids. Moreover, the inhibition matches closely that observed for plant AHAS extracted from native sources (Table 2; refs *14*, *16*, and *19*) in that:

(i) the inhibition by any single amino acid, or any combination, is partial so that there is some residual activity at saturating inhibitor concentrations;

(ii) the potency of inhibition is in the order leucine > valine ≫ isoleucine; and

(iii) there is synergistic inhibition between leucine and either valine or isoleucine.

These observations strongly support the hypothesis that the protein that we have expressed and purified here is the regulatory subunit of *A. thaliana* AHAS.

Reconstitution. Activation of the catalytic subunit by the regulatory subunit is a slow process taking several minutes at 30 °C to reach a maximum. For this reason, reconstitution was performed for at least 8 min, and usually 15 min. The effects of each of the three cofactors on this process have not studied systematically, but inhibition by branched-chain amino acids was usually more pronounced when reconstitution was performed without added cofactors, and then these were added later and the mixture incubated for a further 10 min before adding substrate. There is an hyperbolic dependence (Figure 4) of activity upon the concentration of the regulatory subunit corresponding to a dissociation constant for the complex of 167 nM. This is similar to the value of 70 nM observed for yeast AHAS (*12*).

Full activation requires a substantial molar excess of the regulatory subunit. For example, at the highest concentration of regulatory subunit tested in Figure 4 there is a 27-fold molar excess, and this achieves approximately 70% of the extrapolated limit of full activation. However, this does not imply that reconstitution involves formation of a complex containing multiple molecules of regulatory subunit per catalytic subunit. Rather, it reflects the fact that the concentration of catalytic subunit (13 nM) is low relative to the dissociation constant of 167 nM, and saturation requires a concentration of the regulatory subunit that is well above 167 nM. Estimation of the catalytic/regulatory subunit molar ratio (Figure 5B) by quantitative densitometry after gel-filtration chromatography and SDS−PAGE yielded values of 1.17 and 1.11 in two experiments. The molecular mass of the reconstituted enzyme estimated from this gel-filtration experiment was approximately 548 kDa (Figure 5A), and we suggest that the enzyme is has an $\alpha_4\beta_4$ structure (calculated molecular mass = 470 kDa).

Each of the two repeats of the regulatory subunit is capable of activating the catalytic subunit (Figure 8), although neither is as effective as the intact subunit insofar as higher concentrations were required for any given degree of activation (cf. Figure 4). To find any effect at all of such protein fragments is somewhat surprising and suggests that each folds independently to something approximating the native structure.

Feedback Inhibition. Each of the branched-chain amino acids is inhibitory to reconstituted *A. thaliana* AHAS, with apparent inhibition constants ranging from 0.23 mM (valine) to 1.38 mM (isoleucine). However, the combination of leucine and valine results in a much more potent inhibition (apparent K_i of 12.3 μM). Addition of a low concentration of one of these two amino acids resulted in a very large decrease in the apparent K_i for the other. This synergistic effect suggests that there are two inhibitor-binding sites per regulatory subunit, one binding leucine and the other binding valine. A similar conclusion was reached by Miflin (*14*),

using a partially purified preparation of barley AHAS. On the basis of inhibition constants obtained with combinations of isoleucine with either leucine or valine, we suggest that isoleucine competes for the valine binding site.

Some support for the hypothesis that there are two inhibitor-binding sites was obtained using enzyme reconstituted with each of the two repeats of the regulatory subunit. Reconstitution with repeat 1 confers sensitivity to leucine but not to valine or isoleucine, suggesting that it is repeat 1 that binds leucine. However, AHAS reconstituted with repeat 2 is not inhibited by any of the branched-chain amino acids. This does not rule out the idea that repeat 2 contains the valine/isoleucine binding site and may indicate that the folding of repeat 2 is impaired to the extent that the regulator binding site is not formed properly. It should be noted that each of the repeats, and the entire regulatory subunit, has been expressed with a non-native N-terminal fusion peptide containing the hexahistidine tag. This peptide could alter somewhat the properties of the proteins and may contribute to impaired folding of repeat 2.

Comparison with the N. plumbaginifolia Regulatory Subunit. The sequence of the *A. thaliana* AHAS regulatory subunit closely matches that of *N. plumbaginifolia* (*24*) in that each consists of a chloroplast transit peptide followed by two repeats separated by a linker. The similarity between subunits from the two plant species is particularly high in the repeat regions with more than 80% identical residues. The extent of activation is also similar; we observe 5.4-fold activation (extrapolated to 7.5-fold at saturation), while Hershey et al. (*24*) reported approximately 6-fold activation.

As mentioned earlier, the major difference between the two systems is that the reconstituted *N. plumbaginifolia* AHAS is insensitive to inhibition by branched-chain amino acids, while we have shown effects that closely match those of the native enzyme. There is one difference between the experimental conditions that may be significant and would account for the failure of the reconstituted *N. plumbaginifolia* AHAS to exhibit sensitivity to these effectors. The pH employed by Hershey et al. (*24*) was 7.6, while our studies were conducted at pH 7.0. We observe (Table 3) that the reconstituted *A. thaliana* AHAS shows reduced sensitivity to inhibition at pH 7.5 and almost none at pH 7.8. Possibly, *N. plumbaginifolia* AHAS has a more pronounced response to slightly alkaline pH, so that inhibition is abolished at the pH of 7.6 used by Hershey et al. (*24*).

Relationship to Threonine Deaminase and 3-Phosphoglycerate Dehydrogenase. As noted earlier, the amino acid sequence of the *A. thaliana* AHAS regulatory subunit contains two duplicate regions of ~180 residues (Figure 1, panels B and D). The presence of this duplication, together with the notion that there are separate but interacting binding sites for leucine and valine, invite the speculation that each of these duplicate regions provides one inhibitor binding site. If this hypothesis is correct, there are several striking similarities to threonine deaminase, the enzyme that provides 2-ketobutyrate required by AHAS for the isoleucine branch of the pathway. This enzyme is also regulated by two of the branched-chain amino acids, valine (activator) and isoleucine (inhibitor). Moreover, the three-dimensional structure of *E. coli* threonine deaminase shows that it contains a regulatory domain composed of a pair of repeats with a similar fold (*39*; Figure 9A) and sequence (Figure 9B). Further, Wessel et al. (*25*) have provided evidence that each repeat of *A. thaliana* threonine deaminase contributes a separate binding site for branched-chain amino acids and that there is cooperativity between the two sites. Alignment of the two repeats from the regulatory domain of *E. coli* threonine deaminase with the N-terminal halves of the two duplicate regions of the *A. thaliana* AHAS regulatory subunit (Figure 9B) shows a limited but persuasive homology. We suggest that each of the duplicate regions of the *A. thaliana* AHAS regulatory subunit folds as a separate domain and that the N-terminal halves form structures similar to the two repeats of the threonine deaminase regulatory domain. Each repeat of the plant AHAS regulatory subunit contains an inhibitor-binding site, and the two sites participate in synergistic interactions by virtue of being located near the interface between the two domains.

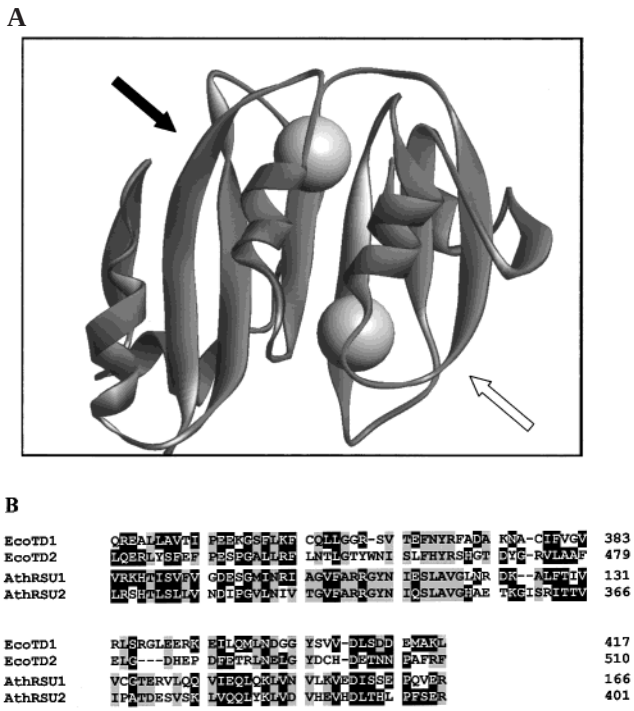

FIGURE 9: Comparison of the *E. coli* threonine deaminase regulatory domain and the *A. thaliana* AHAS regulatory subunit. Panel A shows the structure of the regulatory domain of *E. coli* threonine deaminase (*39*). The first and second repeats are indicated by the black and white arrows, respectively. The spheres show the possible locations where the effectors bind, based on the model of Wessel et al. (*25*). Panel B shows an alignment between the first (EcoTD1) and second (EcoTD2) repeats of the *E. coli* threonine deaminase regulatory domain and the N-terminal regions of the first (AthRSU1) and second (AthRSU2) repeats of the *A. thaliana* AHAS regulatory subunit. Gray shading indicates residues conserved between pairs of repeat sequences (EcoTD1 versus EcoTD2, and AthRSU1 versus AthRSU2). Black shading indicates residues conserved between the *A. thaliana* AHAS regulatory subunit and the *E. coli* threonine deaminase regulatory domain. Conserved residues are defined as in Figure 1.

The structural repeat in the regulatory subunit of AHAS appears to be unique to the plant enzyme with the fungal and bacterial subunit containing only one copy of this sequence. However, the fungal and bacterial enzymes are inhibited solely or preferentially by valine, and there is no evidence of any synergy of inhibition as observed for plant AHAS. Thus, the fungal and bacterial AHAS regulatory

subunit contains only one inhibitor-binding site. On the basis of its preference for valine, this probably corresponds to that formed by the second repeat of the *A. thaliana* AHAS regulatory subunit.

Very recently, Mendel et al. (*40*) have predicted a structure for fungal and bacterial AHAS regulatory subunits, obtained by the fold-recognition algorithm of Fischer (*41*). The structure is based on the regulatory domain of 3-phosphoglycerate dehydrogenase (EC 1.1.1.95). In this enzyme, two regulatory domains from a pair of subunits in the homotetramer are adjacent and form a structure similar to that of a single regulatory domain of threonine deaminase. The allosteric inhibitor, serine, binds to two equivalent sites at the dimer interface (*42*). In the model proposed by Mendel et al. (*40*), the AHAS regulatory subunit corresponds to one of the regulatory domains of 3-phosphoglycerate dehydrogenase and would therefore have to dimerize to create a pair of valine-binding sites. However, earlier work from the same laboratory (*4*) showed that the regulatory subunit of *E. coli* AHAS III, in the absence of its catalytic subunit, is monomeric but binds valine. Thus, we have some reservations about the details of this model, and in particular its reliance on a regulatory subunit dimer to create the binding site for valine, but we accept that it is broadly similar to ours.

It is proposed that the regulatory subunit of *A. thaliana* AHAS and the regulatory domain of threonine deaminase might share a common evolutionary ancestor. Of course, it is also possible that the similarities mentioned above have resulted from convergent evolution. Determination of the three-dimensional structure of the AHAS regulatory subunit of *A. thaliana*, or of any other organism, could assist in distinguishing between these rival hypotheses, but this has not yet been achieved.

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